

Products resulting from cleavage of the interglobular domain of aggrecan in samples of synovial fluid collected from dogs with early- and late-stage osteoarthritis

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Objective—To investigate interglobular domain (IGD) cleavage of aggrecan in dogs with naturally developing osteoarthritis (OA).

Sample Population—Samples of synovial fluid (SF) obtained from 3 cubital (elbow) joints and 3 stifle joints of 4 clinically normal dogs, 24 elbow joints of 12 dogs with early-stage OA, 8 stifle joints of 5 dogs with early-stage OA, and 10 stifle joints of 9 dogs with late-stage OA.

Procedure—Fractions of SF were assayed for total glycosaminoglycan (GAG) content and also subjected to western blot analysis by use of monoclonal antibodies against neopeptides generated by cleavage of the IGD of the aggrecan protein core by matrix metalloproteinase (MMP; BC-14) and aggrecanase (BC-3).

Results—Total GAG content of SF from joints of clinically normal dogs did not differ from that of dogs with early-stage OA. The GAG content of SF from joints of dogs with late-stage OA was significantly lower, compared with GAG content for other SF samples. Aggrecanase-generated fragments were detected in SF from all groups but not in all samples. Matrix metalloproteinase-generated fragments were not detected in any SF samples. In early-stage OA, high-molecular-weight aggrecanase-generated aggrecan catabolites were evident.

Conclusions and Clinical Relevance—GAG content of SF obtained from dogs with late-stage OA is significantly decreased, suggesting proteoglycan depletion of cartilage. Aggrecanases, but not MMPs, are the major proteolytic enzymes responsible for IGD cleavage of aggrecan in canine joints. Analyses of SF samples to detect aggrecanase-generated catabolites may provide an early biomarker for discriminating early- and late-stage OA in dogs. (*Am J Vet Res* 2005;66:1679-1685)

Osteoarthritis (OA) is the most common arthropathy of mammals and a common naturally developing disease of dogs. In dogs, OA is usually secondary to primary conditions, such as dysplasia of the cubital (elbow) joint¹ or rupture of the cranial cruciate ligament.²

Dysplasia of the elbow joint is a common initiating factor for OA in dogs and is a poorly understood dis-

order characterized by developmental abnormalities of the joint surface that most often involve osteochondral fragmentation of the medial coronoid process together with a variable degree of erosion of adjacent articular cartilage and the apposing medial humeral condyle.³ The disorder leads to secondary OA⁴ with further erosion of articular cartilage, synovitis, and periarticular osteophytosis.³ However, at the time of initial examination, dogs are generally skeletally immature and have early-stage disease.

Rupture of the cranial cruciate ligament is the most common disease that affects the stifle (ie, genu) joint of dogs and inevitably leads to the development of OA.² Furthermore, transection of the cranial cruciate ligament is a widely accepted method for experimentally inducing OA in dogs,^{5,6} with full-thickness cartilage lesions associated with OA developing within 3 to 5 years after ligament transection.⁷ However, alterations in cartilage metabolism are evident within 1 to 3 weeks after ligament transection.⁸

One of the earliest events in degradation of articular cartilage is the loss of the large aggregating proteoglycan, aggrecan, from the extracellular matrix. Aggrecan is responsible for the compressive stiffness of articular cartilage because of its large negative charge and subsequent hydrophilic properties.^{9,10} Within the extracellular matrix, aggrecan is bound to hyaluronan at the G1 domain, and this is further stabilized by link protein.¹¹ The extended **interglobular domain (IGD)** that joins the G1 and G2 domains is the main site of proteolytic attack in aggrecan turnover,¹¹ and proteolysis in this region releases most of the molecule from the matrix.

Two major sites of proteolysis have been identified within the IGD. One is between the amino acids asparagine³⁴¹ and phenylalanine³⁴², a site that can be cleaved by **matrix metalloproteinases (MMPs)**. The other is between glutamic acid³⁷³ and alanine³⁷⁴, which is a site that can be cleaved by a novel group of proteases (ie, aggrecanases). The aggrecanases have been identified as members of a class of endopeptidases known as the **a disintegrin and metalloproteinase**

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with a thrombospondin motif (ADAM-TS) family. The principal aggrecanases identified to date are ADAM-TS4 and ADAM-TS5.^{12,13} The MMPs and aggrecanases can both cleave the IGD of aggrecan, but there is considerable debate as to which enzyme has the most important activity in OA.¹⁴⁻¹⁷ However, results of an *in vitro* study¹⁸ in which investigators used bovine and porcine cartilage would suggest that after interleukin-1 stimulation of articular cartilage explants, the IGD epitope is entirely an aggrecanase-generated product for up to 20 days, and only after 28 days of stimulation is the MMP-generated IGD epitope detected.

It is possible to detect the activity of aggrecanase and MMPs on IGD by the use of monoclonal antibodies developed against the new N- and C-terminal peptide sequences. Specifically, antibody BC-3 recognizes the new N-terminus on the aggrecan-core protein generated by the action of aggrecanases, and antibody BC-14 recognizes the new N-terminus generated by the proteolytic action of MMPs.¹⁸⁻²⁰

A study²¹ of cartilage and synovial fluid obtained from humans with knee injuries or OA and from clinically normal subjects revealed 10 species of aggrecan catabolites, 9 of which result from aggrecanase-dependent cleavage. In another study²² conducted by our laboratory group, we detected aggrecanase-generated neopeptides in synovial fluid samples obtained from dogs with experimentally induced OA of the stifle joint. To our knowledge, aggrecanase-generated aggrecan metabolites have not been detected in samples of synovial fluid obtained from dogs with naturally developing OA, and an analysis of the various catabolites released into synovial fluid has not been performed. Because the use of dogs with experimentally induced OA is of importance in research, information on these catabolites would be useful. In addition, it has been suggested from studies in humans with arthritides²¹ and mice with experimentally induced arthritis^{23,24} that IGD cleavage by aggrecanases is evident early in disease, but there is MMP-mediated cleavage of IGD as cartilage destruction progresses. Similar results have been reported for an *in vitro* study²² of cytokine-induced catabolism of bovine nasal cartilage. Furthermore, that *in vitro* study documented that with the onset of MMP activity in late-stage cartilage degradation, the size distribution of aggrecanase-generated (alanine-arginine-glycine-initiating) IGD metabolites was decreased. Considered together, analysis of these results suggests that evaluation of the number and size distribution of aggrecanase- and MMP-generated aggrecan IGD neopeptides may prove useful when assessing progression of cartilage destruction in naturally developing OA. To test this hypothesis, the study reported here was conducted to examine aggrecan metabolites in samples of synovial fluid obtained from clinically normal dogs and dogs with early- and late-stage OA.

Materials and Methods

Sample population—Samples of synovial fluid were collected from joints of 4 groups of dogs. All samples were collected from client-owned dogs examined by a specialist in canine orthopedics at a university-based clinic. Informed

consent for the use of samples in this clinical research was obtained from all owners in accordance with departmental guidelines at the time of the study.

Nonarthritic dogs—Samples of synovial fluid were collected from 6 nonarthritic joints (3 elbow and 3 stifle joints) of 4 skeletally mature dogs (2 Greyhounds, 1 Labrador Retriever, and 1 Rottweiler). Mean age of these dogs was 57 months. These dogs were euthanatized for reasons unrelated to joint disease. Joints were considered free of OA as determined by gross postmortem examination. Samples of synovial fluid were aseptically collected within 30 minutes after the dogs were euthanatized. Samples were centrifuged at 10,000 × g, and supernatant was harvested and stored at -80°C until analyzed.

Dogs with early-stage OA of elbow joints—Samples of synovial fluid were collected from both elbow joints of 12 dogs examined because of lameness in the elbow joint with a confirmed diagnosis of dysplasia and OA of the elbow joint. Dogs ranged from 6 to 30 months of age (mean, 11.4 months) and comprised 7 Labrador Retrievers, 3 Rottweilers, 1 Newfoundland, and 1 Bernese Mountain Dog. All dogs had signs of forelimb lameness, and signs of pain were elicited bilaterally during manipulation of the elbow joints. Radiography (90° flexed mediolateral and craniocaudal projections) of both elbow joints was performed on all dogs, which confirmed bilateral changes consistent with dysplasia and OA. The index elbow joint (ie, elbow joint associated with lameness) was examined arthroscopically by use of a 2.4-mm, 30° arthroscope inserted via a medial portal to additionally confirm the diagnosis of dysplasia of the elbow joint. Before arthroscopy was conducted, synovial fluid was aspirated from both elbow joints for use in cytologic analysis. Excess synovial fluid was centrifuged, harvested, and stored as previously described.

During arthroscopic examination, the degree of chondropathy was graded by use of a region-based scoring system that used a discontinuous ordinal scale with predefined descriptors for each grade (0, normal cartilage; 1, mild fibrillation of the cartilage surface with no exposure of subchondral bone; 2, moderate erosion of cartilage with > 50% of region surface having evidence of cartilage erosion but < 50% exposure of subchondral bone; 3, severe erosion of cartilage with a few islands of cartilage and > 50% exposure of subchondral bone; and 4, extreme erosion of cartilage with no cartilage remaining in the entire region). Regions assessed were the lateral humeral condyle, medial humeral condyle, radial head, medial coronoid process, and semilunar notch of the ulna. An aggregate score for all regions was calculated for each elbow joint.

Dogs with early-stage OA of stifle joints—Samples of synovial fluid were collected from 8 stifle joints of 5 dogs examined because of lameness and signs of pain in the stifle joints associated with cranial cruciate ligament deficiency. Dogs ranged from 16 to 76 months of age (mean, 45.4 months) and comprised 1 German Shepherd Dog, 1 Labrador Retriever, 1 Golden Retriever, 1 Boxer, and 1 English Bullmastiff. These dogs were randomly selected from a prospective cohort of dogs recruited between 1993 and 1995 that were part of studies^{25,26} conducted on the history of dogs with naturally developing cruciate deficiency of the stifle joint.

Briefly, rupture of the cranial cruciate ligament was diagnosed on the basis of results of clinical and radiographic examinations (mediolateral and craniocaudal projections). All dogs underwent lateral parapatellar arthrotomy of affected joints to confirm rupture of the cranial cruciate ligament and enable investigators to categorize the stage of chondropathy. Before surgery, synovial fluid was aspirated from

both stifle joints, centrifuged, harvested, and stored as described previously.

Dogs with late-stage OA of stifle joints—Samples of synovial fluid were collected from 10 stifle joints of 9 dogs with deficiency of the cranial cruciate ligament and late-stage OA. Mean age of dogs at the time of sample collection was 101 months, and the interval since the initial examination because of lameness associated with the stifle joints ranged from 38 to 58 months (mean, 53 months). Dogs comprised 3 Labrador Retrievers, 2 Golden Retrievers, and 1 dog each of 4 other breeds. These dogs were part of the aforementioned cohort of dogs recruited between 1993 and 1995. Dogs were reexamined in 1998 and 1999, approximately 4 to 5 years after the onset of OA.

Preparation and analysis of synovial fluid—Aliquots (0.3 to 1.5 mL) of synovial fluid were added to equivalent volumes of 100mM sodium acetate (pH, 6.8) containing 20mM EDTA, 0.2M 6-amino-hexanoic acid, 10mM benzamidine HCl, and 2mM phenylmethyl sulfonyl fluoride. Density of each sample was then adjusted to 1.5 g/mL by the addition of solid cesium chloride. Samples were centrifuged at 100,000 × g for 48 hours and then divided into 3 equal volumes (A1, A2, and A3) with a mean density of 1.56, 1.48, and 1.43 g/mL, respectively. The A1 fractions contain > 90% of the **glycosaminoglycan (GAG)** content in synovial fluid. The associative conditions also allowed the detection of G1 metabolites and linkage proteins. The remaining fractions (A2 and A3) contain proteoglycans with little or no GAG content, and although some small-molecular-weight, phenylalanine-phenylalanine-glycine-bearing metabolites have been detected in low-buoyant density fractions,¹⁶ our laboratory group has analyzed other fractions and failed to detect any BC-3- or BC-14-positive catabolites (data not shown). Accordingly, only A1 fractions were analyzed.

The A1 fractions were dialyzed exhaustively overnight against 50mM sodium acetate (pH, 6.8) and then dialyzed overnight against purified water^a after which they were lyophilized. Samples were reconstituted in 100mM Tris acetate (pH, 6.5), and GAG content was assayed by use of the dimethylmethylene blue dye binding assay as described elsewhere.²⁷

Chondroitin sulfate and keratan sulfate were removed by the addition of chondroitinase ABC,^b keratanase,^c and keratanase II^d at concentrations of 0.1, 0.1, and 0.001 U/μg of GAG, respectively. Samples were digested at 37°C for 2 hours. Samples were then dialyzed against deionized water overnight, lyophilized, and separated by use of reducing conditions on 4% to 12% Tris-glycine SDS-PAGE gels^e before electrophoretic transfer to nitrocellulose membranes. Loading of samples was standardized by loading an equal amount of GAG (5 μg for BC-3 blots and a minimum of 8 μg for BC-14 blots), which was measured prior to deglycosylation. Immunoblotting of membranes was performed as described elsewhere²⁸ by use of monoclonal antibodies BC-3 (dilution, 1:2,000) that recognized an aggrecanase-generated N-terminal IGD neoepitope,¹⁹ BC-14 (dilution, 1:1,000) that recognized an MMP-generated N-terminal IGD neoepitope,^{19,29} 3B3 (dilution, 1:10,000) that recognized chondroitinase-generated C-6-S stubs on proteoglycans,^{30,31} and 2B6 (dilution, 1:10,000) that detected 4-sulfated stubs of chondroitin sulfate chains on aggrecan.³²

Western blot analysis of cartilage explants—To document that BC-3 and BC-14 can detect aggrecanase- and MMP-generated IGD fragments of canine aggrecan, we conducted extensive experiments that used cultures of cartilage explants obtained from dogs.¹⁸ Briefly, explants of articular cartilage were harvested from macroscopically normal left and right stifle joints (both femoral condyles and tibial

plateau) of skeletally mature dogs euthanatized for clinical reasons unrelated to joint disease. Explants were cultured for 3 days at 37°C in a humidified atmosphere (5% carbon dioxide and 95% air) in Dulbecco modified Eagle medium^h containing 2mM glutamine, 0.1 mg of streptomycin/mL, 50 μg of gentamicin/mL, and 10% fetal bovine serum. Explants were then washed 3 times in serum-free medium, and replicates of an equal wet weight (20 mg) of cartilage were cultured separately in triplicate for 4 days in 1 mL of serum-free medium containing no additives or serum-free medium containing 50 ng of oncostatin M/mL.ⁱ The GAG content in the media was measured as described previously.

Proteoglycan fragments in the conditioned medium were deglycosylated, subjected to SDS-PAGE, and transferred to nitrocellulose membranes as described previously. Loading of gels was standardized by loading on an equal-GAG basis (20 μg), and western blotting with BC-14 and BC-3 was performed as described previously.

Statistical analyses—Differences in GAG content of synovial fluids from the 4 groups were tested by use of the Kruskal-Wallis test with the Dunn multiple comparisons post hoc test. Significance was set at $P < 0.05$. For the largest group (dysplasia of the elbow joint), we investigated the relationships among variables by calculating Spearman rank correlations for GAG content in A1 fractions between paired left and right elbow joints of the same dog, GAG content in A1 fractions and age, and GAG content in A1 fractions and arthroscopic grade of chondropathy.

Results

Staging of OA—Index joints from dogs with dysplasia of the elbow joints were graded arthroscopically on the basis of chondropathy. Scores ranged from 1 to 11 (mean, 4.5) with a maximum possible score of 25, which indicated that all joints were at a relatively early stage of disease. All stifle joints with early-stage OA were inspected at the time of surgical repair and had mild chondropathy. Stifle joints with late-stage OA were not inspected intra-articularly. However, because it was known that OA had been diagnosed 4 to 5 years previously, they were assumed to have late-stage disease.

GAG content of synovial fluid—More than 90% of all the GAG content in synovial fluid was detected in the A1 fraction in all dogs. Accordingly, analysis of synovial fluid GAG was only performed on the A1 samples (Table 1). There was a significant correlation (r_s , 0.775; $P = 0.005$) in GAG content between paired left and right elbow joints. Accordingly, samples from only 1 elbow joint/dog were used for comparison of GAG concentrations in A1 fractions among dogs. For affected dogs, this was always the index joint, and for the other dogs, it was a joint chosen at random.

The A1 fractions of synovial fluid from stifle joints of dogs with late-stage OA had the lowest GAG content, whereas those from stifle joints of dogs with early-stage OA had the highest. In terms of GAG content, none of the affected groups differed significantly from values for the clinically normal dogs. There were significant differences between GAG content of A1 fractions of synovial fluid from elbow joints with dysplasia, compared with content for stifle joints with late-stage OA. Significant differences in GAG content of A1 fractions were also evident between samples collected from stifle joints with early- and late-stage OA.

Table 1—Glycosaminoglycan (GAG) content in A1 fractions of samples of synovial fluid collected from normal joints, cubital (elbow) joints with early-stage osteoarthritis (OA), stifle joints with early-stage OA, and stifle joints with late-stage OA from dogs.

GAG content ($\mu\text{g/mL}$)	Normal joints (4)	Elbow joints with early-stage OA (12)	Stifle joints with early-stage OA (5)	Stifle joints with late-stage OA (9)
Mean \pm SD	22.8 \pm 30.7	31.6 \pm 16.0	48.4 \pm 21.5	8.9 \pm 3.0
Median	10.5	27.1 ^a	51.6 ^a	8.2 ^b
Range	2.2–83.6	11.0–74.1	21.1–69.2	5.5–15.3

Numbers in parentheses are the number of joints from which samples were collected.
^{a,b}Values with different superscript letters differ significantly ($P < 0.05$; Dunn multiple comparisons test).

Western blot analysis of synovial fluid—The A1 fraction in 1 of 6 samples of synovial fluid collected from joints of nonarthritic dogs had a GAG content of only 2.2 $\mu\text{g/mL}$; we considered this insufficient for additional analysis. We detected BC-3–positive fragments in 3 of the 5 remaining samples of synovial fluid (Figure 1). A large fragment of approximately 250 kd was evident in 1 sample, and relatively larger quantities of a fragment migrating at approximately 148 kd were evident in all samples. None of the 5 samples of synovial fluid collected from the clinically normal dogs revealed BC-14–positive bands (data not shown). None of the BC-3–positive metabolites < 110 kd reacted with 3B3.

One sample of synovial fluid collected from an elbow joint with early-stage OA had insufficient GAG content for meaningful analysis and was excluded from additional testing. Of the 23 remaining joints with elbow dysplasia and early-stage OA, all had positive-staining results, with a band at approximately 148 kd. In addition, 17 of 23 had a band at approximately 250 kd, and 9 of 23 had an intermediate band at approximately 200 kd. There was not an apparent association between arthroscopic grade of chondropathy and number of BC-3–positive bands. None of the A1 fractions of synovial fluid collected from elbow joints of dogs with early-stage OA had BC-14–positive bands (data not shown).

Of the 8 samples of synovial fluid collected from stifle joints with early-stage OA attributable to rupture of the cranial cruciate ligament, all had BC-3–positive staining with a band at approximately 148 kd. In addition, 6 of 8 had a BC-3–positive band at 200 kd, and 4 of 8 had a BC-3–positive band at 250 kd. Western blot analysis performed by use of 3B3 revealed results similar to those for synovial fluids obtained from elbow joints with early-stage OA in that none of the BC-3–positive metabolites < 110 kd reacted with 3B3.

Western blots for BC-3 were compared with western blots for 3B3 and 2B6 (Figure 2). Evaluation revealed that not all BC-3–positive fragments had positive staining results for 3B3; small (< 110 kd) BC-3–positive fragments were devoid of chondroitin sulfate. There also was weak staining of 3B3–positive metabolites of slightly > 250 kd; these did not appear to be BC-3–positive metabolites. In addition, larger fragments had 2B6–positive staining, but fragments

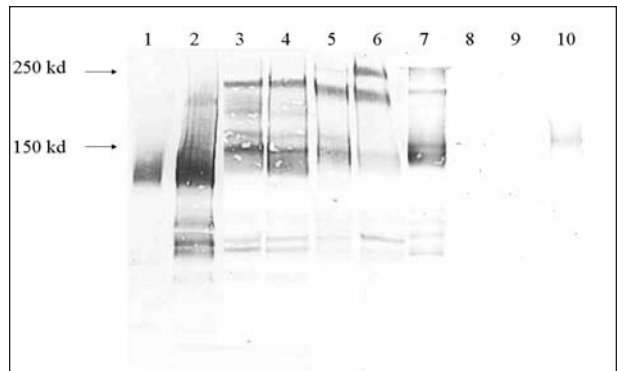


Figure 1—Western blots of representative deglycosylated A1 density-gradient fractions of samples of synovial fluid obtained from joints of dogs. Notice the bands that represent positive staining for BC-3. Lanes were as follows: 1 and 2, normal joints; 3 and 4, cubital (elbow) joints with early-stage osteoarthritis (OA); 5 and 6, stifle joints with early-stage OA; and 7, 8, 9, and 10, stifle joints with late-stage OA. Positive staining for BC-3 indicates aggrecanase-generated aggrecan catabolites with a new N-terminus in the interglobular domain (IGD).

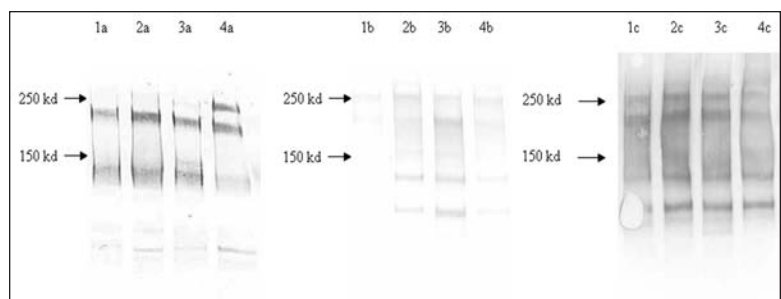


Figure 2—Western blots of deglycosylated A1 fractions of samples of synovial fluid obtained from 4 representative stifle joints with early-stage OA. Lanes were as follows: 1a to 4a were stained to reveal BC-3, 1b to 4b were matching blots stained to reveal 2B6, and 1c to 4c were matching blots stained to reveal 3B3. Positive staining for BC-3 indicates aggrecanase-generated aggrecan catabolites with a new N-terminus in the IGD, whereas 2B6 detects 4-sulfated stubs of chondroitin sulfate chains on aggrecan, and 3B3 detects chondroitinase-generated C-6-S stubs on proteoglycan.

< 110 kd did not react with 2B6, which indicated a lack of attachment domains for chondroitin sulfate.

Only 2 A1 fractions for the 8 samples of synovial fluid collected from stifle joints with late-stage OA had BC-3–positive bands. One of these only had a band at 148 kd, but the other had bands at 148, 200, and 250 kd. None of the samples had BC-14–positive bands. The gel of the western blot that used BC-3 was probed again by the use of monoclonal 3B3 to detect chondroitin sulfate epitopes, and this revealed weak but positive staining of all lanes for metabolites > 110 kd.

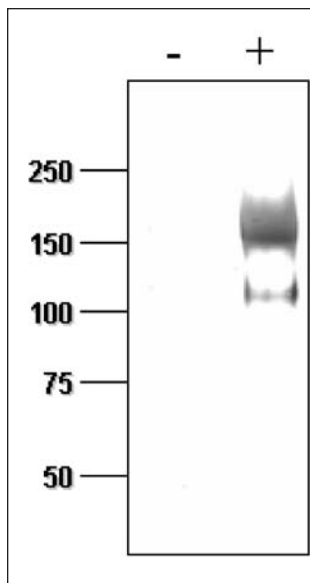


Figure 3—Western blot of conditioned media after explants of canine articular cartilage were cultured without (–) or with (+) oncostatin M (50 ng/mL). Notice that BC-14–positive bands are evident in media of explants cultured with oncostatin M. Numbers on left side represent molecular weight in kilodaltons.

Western blot analysis of cartilage explants—

Results of these experiments have been reported elsewhere.¹⁸ Briefly, we documented the ability of BC-14 to detect the MMP-generated IGD N-terminus because none of the samples of synovial fluid had positive staining for BC-14. All media from unstimulated explants and explants stimulated with oncostatin M had BC-3–positive bands (data not shown). A few cultures had 2 BC-14–positive bands (Figure 3). This confirmed that BC-14 is able to detect the MMP-generated IGD N-terminus.

Discussion

Samples used in the study reported here were obtained from clinically normal and OA-affected dogs examined at a university-based veterinary

orthopedic referral service. Samples were selected at random from larger sample banks and were considered representative of the respective patient groups. Nevertheless, it is probable that these samples were not entirely representative of dogs with these types of joint disease. For example, in the experience of one of the investigators (JFI), it is not always possible to retrieve a sufficient amount of synovial fluid from elbow joints of dogs with dysplasia of the elbow joint or from stifle joints of dogs with chronic OA in those joints, although this is rarely the case for stifle joints with early-stage OA. Therefore, it is likely that there was some inherent bias in this study toward dogs that generated larger volumes of synovial fluid.

In the evaluation of aggrecan fragments in A1 fractions of synovial fluid, we considered each joint as the experimental unit. This seems a valid approach for a localized disease such as OA. However, there is also a possibility that each dog may have had a tendency to generate particular sizes of aggrecan fragments. Subjective review of our data indicated that paired samples of synovial fluid obtained from a dog can have marked differences in patterns of BC-3–positive catabolites, which suggests that the status of a specific joint influences aggrecan catabolism. Nevertheless, additional studies are required to determine whether there are inherent differences in aggrecan catabolism among dogs.

Other background and disease variables should also be considered as potential confounders in this study. We were unable to find a significant relationship between GAG content in A1 fractions from elbow

joints with dysplasia and age of dogs. In another study²⁶ conducted by our laboratory group, synovial fluid from 58 dogs with OA of a stifle joint also did not have a significant relationship between age and GAG content. In the study reported here, our sample size for each group was too small to consider the effect of age on size of aggrecan fragments in A1 fractions. However, the effect of age on MMP- and aggrecanase-generated IGD N-terminal degradation products in intervertebral disks and articular cartilage of humans has been evaluated.³³ Analysis of results of that study revealed that MMP-generated G1 content increases in articular cartilage as patients grow older, whereas aggrecanase-generated G1 content increases until humans become adults and then remains steady thereafter. This may suggest that MMPs are more important in older people. However, it must be remembered that the MMP-generated G1 neopeptide can be readily generated from the aggrecanase G1 fragment; thus, the increase in MMP-generated G1 content with increasing age may represent C-terminal truncation of the already formed aggrecanase-generated G1 fragment. There appears to be no other published evidence from studies in humans or other animals concerning the manner in which the relative contribution for these 2 enzymatic cleavages changes with age.

In the study reported here, we documented that aggrecanases were active in IGD cleavage in some of the normal joints and all of the osteoarthritic joints with early-stage disease. The most common aggrecan species migrated to a position of approximately 148 kd, which is consistent with BC-3–positive fragments released from unstimulated canine cartilage in vitro (unpublished data) and those from A1 fractions of synovial fluid collected from dogs with experimentally induced OA of the stifle joints.²² It is interesting that only a minority of samples of synovial fluid from dogs with late-stage disease had BC-3–positive bands, despite adequate quantity of total GAG as determined by use of the dimethylmethylene blue assay and western blot analysis for the 3B3 epitope of chondroitin sulfate. This highlights that BC-3 is not merely a surrogate for total GAG content and may well be a marker of disease stage (ie, early-stage disease). However, in elbow joints, we did not observe a significant relationship between GAG content in the A1 fraction of synovial fluid and arthroscopic grade of chondropathy, although the numbers included in this study were small.

The BC-3–positive aggrecan metabolites detected in the study can be compared with those detected in synovial fluid obtained from the knees of humans²¹ and from dogs with experimentally induced OA of the stifle joints.²² Analysis of synovial fluid obtained from human knees would suggest that the large 250-kd fragment is the result of cleavage between alanine-arginine-glycine³⁷⁴ and glutamic acid-leucine-glutamic acid¹⁵⁴⁵ or alanine-arginine-glycine³⁷⁴ and glutamic acid-glutamic acid-glutamic acid¹⁷¹⁴ of the mature secreted aggrecan molecule.²¹ Comparison to the canine aggrecan sequence suggests that the equivalent fragments are likely to be alanine-arginine-glycine³⁹⁴ to glutamic acid-leucine-glutamic acid¹⁴⁶¹ and alanine-arginine-glycine³⁹⁴ to glutamic acid-glutamic acid-glutamic

acid¹⁶⁶⁷. However, confirmation of this would require double-immunoreactivity experiments conducted by use of SDS-PAGE. In the synovial fluid obtained from elbow joints with early-stage OA and stifle joints with early- or late-stage OA, the appearance of large (approx 250 and 200 kd) BC-3-positive metabolites was common, whereas weak staining for the 250-kd metabolite was seen in only 1 sample of synovial fluid obtained from a normal joint. These large 250- and 200-kd metabolites could have been the result of degradation of a pool of aggrecan not degraded in normal joints, or it could represent appearance of newly synthesized aggrecan subsequently degraded by aggrecanases. Examination of western blots that involved the use of 3B3 also revealed weak staining for 3B3 metabolites slightly larger than 250 kd that did not appear to be BC-3-positive metabolites. This also may have been newly synthesized aggrecan.

On the basis of the small number of samples examined in the study reported here, it appears that there is a tendency for the appearance of smaller aggrecan fragments in late-stage OA. This may be explained by C-terminus truncation via the activity of MMPs.²¹ However, additional studies are required to confirm this observation.

We were not able to document any BC-14-positive bands in any of the A1 density-gradient fractions examined in this study, despite the fact that BC-14 was able to detect MMP-digested aggrecan from canine cartilage in this study and another study.²² However, analysis of results for our in vitro studies^{4,8} of unstimulated cartilage explants obtained from dogs indicates that all cartilage explants released BC-3-positive aggrecan into the culture medium but that only a small number released BC-14-positive metabolites into the culture medium after stimulation with oncostatin M. Furthermore, the BC-14-positive metabolites seen in BC-14-positive, oncostatin M-stimulated culture medium of canine cartilage explants appeared as bands of the same size, similar to results in another study²² that involved MMP digestion of porcine and bovine aggrecan and those released from bovine nasal cartilage in response to interleukin-1 and inhibited by MMP inhibitors. In other studies^{18,34} conducted by our laboratory group, we documented by use of dilution curves of MMP-digested aggrecan in other species (porcine and bovine) that BC-14 can detect the MMP-generated IGD N-terminus when as little as 3% of the loaded sample initiates with this sequence.

In the study reported here, we documented that aggrecanases are primarily responsible for the cleavage of aggrecan in the elbow and stifle joints of dogs with naturally developing OA. This degradative activity appears to persist into late-stage OA of the stifle joint. Inhibition of aggrecanase activity within affected joints may provide a useful therapeutic target for dogs with OA.

- a. Milli-Q water, Millipore (UK) Ltd, Watford, UK.
- b. Chondroitinase ABC, Sigma Chemical Co, Poole, UK.
- c. Keratanase, Seikagaku America, Falmouth, Mass.
- d. Keratanase II, Seikagaku America, Falmouth, Mass.
- e. Novex, Frankfurt, Germany.
- f. Little CB, Cramp J, Curtis CL, et al. Canine articular cartilage

degradation: the role of catabolic cytokines (abstr). *Trans Orthop Res Soc* 2003;28:699.

- g. Curtis CL, Cramp J, Vaughan-Thomas A, et al. Differential effects of canine cartilage to cytokine stimulation and n-3 polyunsaturated fatty acids supplementation versus human and bovine cartilage (abstr). *Trans Orthop Res Soc* 2004;29:626.
- h. Dulbecco modified Eagle medium, Gibco-BRL, Paisley, UK.
- i. Human recombinant oncostatin M, Preprotech, London, UK.

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